

EDITORIAL COMMENT

Inorganic Nitrate Therapy Improves Doxorubicin-Induced Cardiomyopathy

A New Window for an Affordable Cardiovascular Therapy for Everyone?*

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The anthracycline doxorubicin (DOX) is a potent and effective antineoplastic antibiotic agent widely used in the treatment of a broad range of forms of cancer. The clinical use of DOX is limited by cardiotoxicity, which increases dose dependently and may lead to dilated cardiomyopathy and clinical manifestations of congestive heart failure up to 10 to 15 years after the cessation of DOX chemotherapy in as many as 30% to 40% of patients treated with this drug (1). It is well established that cardiac oxidative stress from nicotinamide adenine dinucleotide phosphate oxidases (2) and mitochondrial sources (3) contributes substantially to

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DOX-associated complications and that early disturbances in mitochondrial biogenesis and damage of mitochondrial deoxyribonucleic acid represent other hallmarks of DOX-mediated cardiotoxicity (4). Notably, a role for oxidative stress is supported by studies in genetically modified mice indicating that deficiency of antioxidant enzymes such as glutathione peroxidase-1, an important hydrogen peroxide-degrading enzyme, increased susceptibility to DOX-induced cardiotoxicity (5), whereas overexpression of manganese superoxide dismutase, an essential antioxidant enzyme in mitochondria, prevented these adverse effects of DOX (6). In an alternative concept for the mechanism of DOX-induced cardiotoxicity, independent of oxidative

stress, it was recently hypothesized that DOX-dependent modulation of the phosphoproteome, cardiac transcriptional reprogramming, and especially inactivation of adenosine monophosphate-activated protein kinase (AMPK) and the creatine kinase, which are 2 key kinases involved in cardiac energy metabolism (7,8), might play a causal role. Because many kinases and phosphatases, including AMPK, are activated or inhibited by reactive oxygen and nitrogen species, however, these 2 hypotheses should not necessarily be seen as antagonistic, as low levels of reactive oxygen species (below the possibility of detection) under long-term DOX therapy might lead to modifications in the phosphorylation pattern of these enzymes, modulating their kinase and phosphatase activity.

Improvement of Doxorubicin-Induced Cardiotoxicity by Inorganic Nitrate Therapy

In this issue of the *Journal*, Zhu et al. (9) show in an elegant and convincing way that the coadministration of inorganic nitrate significantly diminishes DOX-induced cardiotoxicity and associated cardiomyopathy in an experimental animal model, as demonstrated by reduced impairment in ejection fraction and fractional shortening. Furthermore, inorganic nitrate therapy was associated with lesser changes in systolic pressure, parameters of systolic and diastolic dysfunction, heart rate, mean aortic blood pressure, and end-diastolic pressure in DOX-treated animals. As expected, nitrate therapy increased the plasma levels of nitrate, nitrite, and nitric oxide (NO) products (including nitrosyl-iron species and nitrosothiols), a feature that was interestingly shared by DOX treatment. However, combination of both treatments was not additive with respect to nitrate and NO product levels, while nitrite plasma levels were actually lower in animals treated with nitrate and DOX compared with those treated with nitrate alone.

Inorganic nitrate therapy almost completely prevented DOX-mediated cardiomyocyte necrosis (on trypan blue staining), and cardiomyocyte apoptosis (on terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling assay) induction was partially reduced in the nitrate + DOX group, while the function of mitochondrial complex I (but not complex II) of the respiratory chain was largely impaired by DOX treatment and normalized by nitrate therapy. In accordance with the oxidative stress concept of DOX-induced cardiotoxicity, the level of cardiac lipid peroxidation (malondialdehyde and 4-hydroxynonenal) was dramatically increased by DOX treatment and normalized by nitrate therapy. Besides these fascinating data, another major advance of the study is that 2 different administration routes for the inorganic nitrate were tested, namely, oral administration in drinking water and subcutaneous infusion by osmotic minipumps using similar doses. Although subcutaneous therapy with inorganic nitrate increased plasma nitrate levels to a similar extent compared with oral nitrate administration, there was no increase in plasma nitrite levels and only minor effects on cardiac function in response to DOX

*Editorials published in the *Journal of the American College of Cardiology* reflect the views of the authors and do not necessarily represent the views of JACC or the American College of Cardiology.

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treatment. The fact that oral, but not subcutaneous, treatment with inorganic nitrate had protective effects appears to indicate that bioconversion by microorganisms in the mouth and/or the gastrointestinal tract is required for the cardioprotective effects of inorganic nitrate.

Bioactivation of Inorganic Nitrate and Nitrite to Vasodilatory Species

There is growing evidence for the beneficial effects of inorganic nitrate and nitrite therapy on cardiovascular diseases and complications (10). Most of these studies support the concept that a bioactivation of the inorganic nitrate to nitrite, which is more easily converted to the vasodilator NO or a related species (e.g., nitrosothiols) by deoxygenated hemoglobin (11,12) or in an acidic milieu (e.g., in the stomach or in the mitochondrial intermembrane space), is required. Because inorganic nitrate is an inert compound, its reduction to nitrite is not easily performed by many enzymatic systems (13–15). It is well established that microorganisms such as bacteria and fungi display enzymatic activities reducing inorganic nitrate, leading to its effects on blood pressure and platelet function in different systems and models (16). For mammalian tissues or homogenates, a nitrate-reducing activity has been postulated for several years. In 2008, xanthine oxidoreductase was identified as a nitrate-reducing enzyme *in vitro*, *ex vivo*, and *in vivo* (17). However, to what extent the bacterial and mammalian pathways contribute to the overall conversion of inorganic nitrate to nitrite, and subsequently to the bioactivation of nitrite to the vasodilator NO, has not been investigated so far (Fig. 1). In the present study, the evidence that despite the similar plasma nitrate levels obtained via the 2 separate administration routes, only the oral treatment resulted in an appreciable improvement of cardiac function under DOX therapy supports the concept that a gastrointestinal bioactivation of nitrate is a necessary step. Future studies should further elucidate the importance of both bioactivation pathways for the cardiovascular protective effects of inorganic nitrate therapy. Because treatment with the xanthine oxidoreductase inhibitor allopurinol *per se* displays beneficial effects in cardiovascular disease, this intervention cannot be used to investigate the contribution of this enzyme to the bioactivation of inorganic nitrate. However, the fact that tungsten treatment leads to replacement of the molybdenum in xanthine oxidoreductase and results in an inactive enzyme suggests a possible strategy to investigate this issue.

Is Nitrite and Nitrate Treatment the Next-Generation Therapy for Cardiovascular Diseases?

A highly protective profile of dietary inorganic nitrate, as demonstrated by reductions of mean arterial pressure, renal function, cardiac hypertrophy, and fibrosis as well as by the normalization of oxidative stress markers (asymmetric dimethylarginine, malondialdehyde, isoprostane, and 8-oxo-deoxyguanosin) has been described in salt-induced hyper-

tension (18). Very similar protective effects were reported for endothelial NO synthase knockout mice displaying features of metabolic syndrome and receiving inorganic nitrate therapy (19). In this model, inorganic nitrate lead to decreases in triglycerides, visceral fat, blood glucose, and glycosylated hemoglobin and to an increase in insulin production. Furthermore, another battlefield for inorganic nitrate and nitrite therapy may be, for example, the prevention of ischemia-reperfusion injury (10,20).

Remaining Pieces in the Puzzle

Although the concept that inorganic nitrate and nitrite may mediate cardiovascular protection is quite attractive, there are still some caveats and open questions. Nitrite is a very weak activator of recombinant soluble guanylyl cyclase in the presence of mitochondria (21). The required concentration for maximal activation of the purified soluble guanylyl cyclase was 1 $\mu\text{mol/l}$ for the direct NO donor diethylamine NONOate and 10 mmol/l for nitrite. Moreover, it is known that inorganic nitrate but also nitrite are very weak vasodilators in isolated aortic ring segments. Therefore, it is hard to understand how a 2-fold change in plasma nitrite levels can be responsible for such a dramatic change in NO levels in the present animal model.

Another drawback could be the formation of nitrosamines in response to oral inorganic nitrate and to a much higher extent oral inorganic nitrite therapy (13,14), which are known potent inducers of cancer. This side effect could be avoided by nonoral administration routes of inorganic nitrate and nitrite. However, because the study of Zhu et al. (9) provides good evidence that nonoral therapy may be less efficient (or even devoid of beneficial effects), another future challenge could be to identify new routes for the delivery of inorganic nitrate and nitrite that are safe yet effective. Methemoglobinemia formation may also represent another important side effect of inorganic nitrate and nitrite therapy, which can probably only be avoided by an optimal dosing regimen (13).

Perspectives

The results presented in this issue of the *Journal* provide a new attractive hypothesis for DOX toxicity based on the observation that DOX treatment increases 4-hydroxynonenal, a toxic aldehyde (6). This concept is supported by our own observations that mice with homozygous deficiency in mitochondrial aldehyde dehydrogenase-2 (ALDH-2) are more susceptible to DOX-induced cardiac and vascular damage (22). Noting that ALDH-2 catalyzes the detoxification of 4-hydroxynonenal (Fig. 1), inhibition of this enzyme would result in a deleterious feedback mechanism. Considering the recent findings on increased susceptibility of ALDH-2-deficient mice to ischemic damage in experimental myocardial infarction and inactivation of AMPK by 4-hydroxynonenal (23), there seems to be an important link between the observations on DOX-induced AMPK inacti-

vation (7,8) and a feasible explanation for the highly protective effects of inorganic nitrate and nitrite in ischemia-reperfusion models. Zhu et al. (9) provide clear evidence that inorganic nitrate prevents mitochondrial dysfunction and mitochondrial oxidative stress, which provides a mechanistic background to the beneficial effects of inorganic nitrate on DOX-induced cardiotoxicity observed in previous reports. In addition, NO, a major mediator of the beneficial effects of inorganic nitrate, plays an important role in ischemic pre-conditioning, an important protective mechanism in which mitochondrial pathways play a crucial role. Taking into account all of these observations, it is recommended to explore the effects of inorganic nitrate and nitrite on mitochondria in more detail and to explore the role of the mitochondrial protein ALDH-2 as well as the AMPK, which contributes to regulation of mitochondrial

biogenesis and energy metabolism. The concept of NO-mediated protective effects of inorganic nitrate and nitrite may not be as simple as postulated in most of the previous publications on this topic, because classic NO donors (e.g., NONOates, sodium nitroprusside) do not possess similar beneficial properties, especially under long-term therapy.

There is a great need for clinical proof of effectiveness of these therapeutic interventions in humans, which should be based on large outcome-directed clinical trials. If these trials are successful and prove the effectiveness of inorganic nitrate and nitrite therapy in various cardiovascular complications, this would offer a new window for cheap and affordable cardiovascular therapy for everyone, a highly desirable result.

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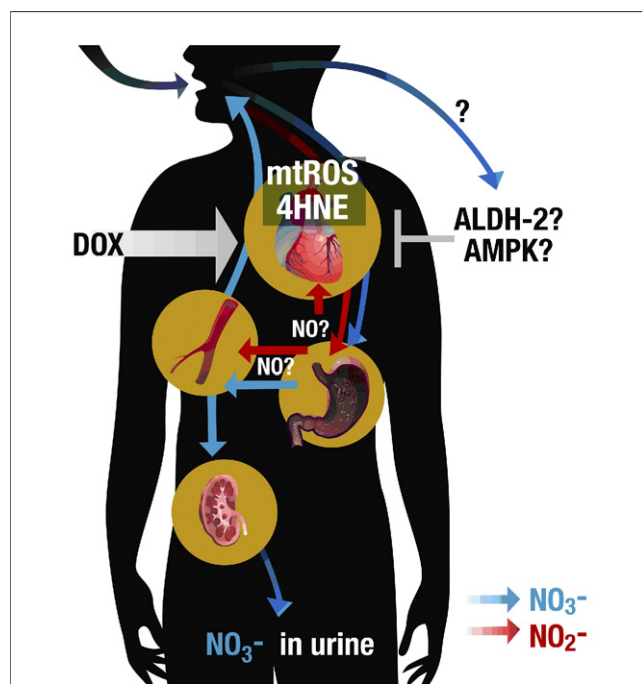


Figure 1 Metabolic Pathways of Nitrite and Nitrate Affecting Doxorubicin-Induced Cardiotoxicity

In the oral cavity, the bioactivation of the more stable nitrate (NO_3^-) to nitrite (NO_2^-) requires an initial reduction process by symbiotic bacteria and by the gastric mucosa (and, according to recent reports, also by xanthine oxidoreductase). Nitrite, in turn, can be further reduced to nitric oxide (NO) within mitochondria by components of the respiratory chain, by acidic disproportionation in the stomach, or in the intermembrane space of mitochondria or by the xanthine oxidoreductase (e.g., under the condition of hypoxia leading to vasodilation and accordingly blood pressure reduction). It should be noted that nitrate infusion in the study by Zhu et al. (9) had no protective effects. Doxorubicin (DOX) treatment causes a cardiomyopathy characterized by systolic and diastolic dysfunction, increased mitochondrial damage and formation of oxygen-derived free radicals, and increased lipid peroxidation with enhanced formation of toxic products such as malondialdehyde and 4-hydroxynonenal (4HNE). Nitrate therapy reversed all of these abnormalities and reduced cardiomyocytic necrotic and apoptotic cell death. It is also likely that some of the beneficial effects of nitrate therapy involves an activation of protective enzymes such as aldehyde dehydrogenase-2 (ALDH-2) and adenosine monophosphate-activated protein kinase (AMPK). mtROS = mitochondrial reactive oxygen species.

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Key Words: cardioprotection ■ doxorubicin ■ heart failure ■ inorganic nitrate ■ mitochondria.